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EFFECTS OF DICHLORVOS AND CHARACTERIZATION OF MICROSOMAL NADPH-CYTOCHROME P450 AND NADH-FERRICYANIDE REDUCTASE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) LIVER

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Abstract

The kinetics of oxidation of rainbow trout (*Oncorhynchus mykiss*) NADPH-cytochrome P450 reductase (NCPR) by cytochrome-c and NADH-ferricyanide reductase (NFR) by $K_3Fe(CN)_6$ were studied. NCPR and NFR activities from liver microsomes of rainbow trout (*Oncorhynchus mykiss*) had a specific activity of 0.0246 and 0.0350 nmoles/min/mg protein, respectively. The maximal rate of NCPR reaction was found at cytochrome-c concentrations above 2.1 mM and in the presence of cyt c. V_{max} and K_m were 0.0083 nmoles/min/mg and 1.14 mM. The maximal rate of NFR reaction was at $K_3Fe(CN)_6$ concentrations higher than 2.0 mM and in the presence of $K_3Fe(CN)_6$. V_{max} and K_m values were 1.04 nmoles/min/mg and 0.352 mM, respectively. The inhibition by dichlorvos (DDVP) on NCPR and NFR activity was not exhibited over the range 1.0-5.0 mM DDVP.

Introduction

DDVP is an organophosphate medicine, used to treat farmed salmon infested with ectoparasitic crustacean sea lice (Murison et al., 1997; McHenery et al., 1997). A clinical pharmacological study was carried out to evaluate the pharmacokinetics of metrifonate

and its active metabolite DDVP (Heinig et al., 1999). Chronic DDVP treatment and early electrophysiological changes were investigated in three series of experiments (Desi et al., 1999). DDVP is usually used as a veterinary medicine to protect farmed salmon against

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salmon lice and as an aerosol against cat and dog fleas in some countries. Although DDVP has been used for 40 years, considerable uncertainties remain about whether or not it is implicated in phase I detoxification enzymes and the wider consequences of its use. Only the *in vitro* effects of DDVP (0.2-100 mg/l) on total amylolytic, sucrase and protease activities of intestinal mucosa have been investigated (Golovanova et al., 1999).

Cytochromes P450 comprise a superfamily of ubiquitous monooxygenases associated with the metabolism of a broad group of structurally unrelated compounds, including drugs, chemical carcinogens, environmental pollutants and endogenous substrates such as steroids, fatty acids and prostaglandins (Nebert and Negishi, 1982; Waterman et al., 1986).

The process of metabolism of these and other xenobiotics converts lipophilic compounds to more polar molecules either by modification or conjugation reaction. P450 inhibitors can be broadly classified into two structural groups. One group is related to structural similarity of the inhibitor to that of the physiological substrate, which is a steroid-based inhibitor of aromatase, lanosterol-14 α -demethylase, cholesterol-7 α -hydroxylase, and aldosterone synthase (Shimizu et al., 1995; Trzaskos et al., 1995). The other group consists of compounds that can coordinate to the sixth position of the hem iron (Ibrahim and Buzdar, 1995; Baroudi et al., 1996). Several mechanism-based inhibitors have been developed for specific enzymes. High affinity and specificity with a biological target are desirable attributes of potential therapeutic agents, as well as its ability to function as a mechanism with a low partition number (Brueggemeier, 1994; Johnson et al., 1995). On the inactivation of hepatic microsomal P450 by DDVP (dichlorvos; 2,2-dichlorovinyl dimethyl phosphate), the vinylphosphate phosphorylating reagent has received little attention as a potential hem binding ligand for the design of P450 inhibitors. The vinylphosphates are sensitive to phosphorylation and may be readily phosphorylated by the P450 system to reactive intermediates.

This paper deals with an *in vitro* study in trout livers of the main enzymes catalyzing functionalization reactions such as cytochrome P450 related NCPR (NADPH-cytochrome P450 reductase) and NFR (NADH-ferricyanide reductase). There is no clear evidence that DDVP affects the liver of rainbow trout (*Oncorhynchus mykiss*). In this study, we characterize and report on the kinetics of microsomal NCPR and NFR in rainbow trout and the effects of DDVP on enzyme activity. Until now, there has been no published information on the development of resistance to DDVP by NCPR and NFR

Materials and Methods

Chemicals. Folin and Ciocalteu's phenol reagent, KCl, EDTA, butylated hydroxytoluene (BHT), trizma base, bovine serum albumin (BSA), NADH, NADPH, polyethylene glycol (PEG) 6000, sodium cholate, acetic acid, KHPO₄, K₂HPO₄, cytochrome-c, glycerol, dithiothreitol and K₃Fe(CN)₆, were purchased from Sigma. Nitrogen and CO were obtained from Habas, Istanbul, Turkey. All chemicals were of analytical grade and purchased commercially. DDVP (dichlorvos, 2,2-dichlorovinyl dimethyl phosphate, 98.0% purity) was purchased from Chemex International Corporation Ltd., Wancai, Hong Kong.

Partially purified cytochrome P450, NCPR and NFR. Rainbow trout (*Oncorhynchus mykiss*, 203 \pm 4.0 g, total body length 25 \pm 0.63 cm) were taken from cages suspended in the Black Sea at Öz Balıkçılık (Samsun, Turkey) and fed commercial products from Pinar Yem Ltd. (Istanbul, Turkey). The fish were removed from the cages, killed by decapitation, and the livers were excised immediately. After removal of the gall bladder, the livers were dropped into liquid nitrogen and stored until use in polypropylene tubes so as not lose enzyme activity.

Subsequent steps were carried out at 0-4°C. The livers were washed several times in cold double distilled water and 1.15% KCl. Microsomes were prepared in a resuspension buffer (0.1 M Tris-acetate buffer, pH 7.4 containing 0.1 M KCl, 1.0 M EDTA, and 2.3 x

10⁻⁵ M BHT) with some modification of an earlier procedure (Theodore et al., 1974; Iyanagi et al., 1978). After elimination of cell debris and mitochondria, microsomes were obtained by centrifugation (10,000 x g for 30 min supernatant and 105,000 x g 90 min). The microsomal pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4 containing 1.0 mM EDTA and 2.3 x 10⁻⁵ M BHT. The mixture was stirred twice for 60-second intervals, treated for 30 seconds in a sonicator at full out-put (10-12 amplitude microns) and centrifuged at 105,000 x g for 60 minutes. The phosphate-extracted microsomal suspension was mixed with 0.1 M Tris-acetate buffer, pH 7.4 containing 0.1 M KCl, 20% glycerol, 1.0 mM mercaptoethanol, 1.0 mM EDTA, and 2.3 x 10⁻⁵ M BHT. The fractions precipitating from 0-4%, 4-6%, 6-8%, 8-10% and 10-13% PEG were separated by centrifugation at 10,000 x g for ten minutes. The 8-10% PEG fraction includes the richest cytochrome P450 while the 10-13% PEG fraction contains NCPR and NFR in the purification of cytochrome P450.

Determination of protein. Microsomal and cytosolic protein was determined by the method of Lowry et al. (1951) with slight modifications using bovine serum albumin (BSA) as a standard at 660 nm. A standard curve of 0-100 µg/mL BSA was used to calculate protein amounts in samples.

Assay of cytochrome P450. Total cytochrome P450 was determined in a microsomal suspension by carbon monoxide-difference spectra of dithionite-reduced microsomes using an extinction coefficient of 91/mM/cm between 450 and 490 nm (Omura and Sato, 1964).

Assay of NCPR. NCPR activity in the microsomal suspension was determined spectrophotometrically by measuring the rate of reduction of an artificial substrate, cytochrome-c, at 550 nm (Masters et al., 1967). The mixture contained 0.3 M potassium phosphate buffer (pH 7.7), cytochrome-c (1.1 mM), 3.9 mM NADPH and microsomal enzyme in a final volume of 1.0 ml. The reaction was initiated by the addition of NADPH and followed for a few minutes at 550 nm at

25°C. Reductase activities were calculated using the extinction coefficient of 19.6/mM/cm for the difference in absorbance between 550 and 450 nm. One unit of reductase is defined as that causing the reduction of 1.0 mole of NADPH per minute under the conditions described above.

Assay of NFR. NFR in the microsomal suspension was determined spectrophotometrically by measuring rate of reduction of ferricyanide which acted as an electron acceptor at 420 nm (Masters et al., 1967). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 3 mM NADH, 5 mM potassium ferricyanide and microsomal enzyme in a final volume of 1.0 ml. The reaction was started by addition of NADH at 25°C. The enzyme activity was calculated using the extinction coefficient of 1.02/µM/cm for difference in absorbance at 420 nm. One unit of enzyme activity is defined as the amount of enzyme causing the reduction of 1.0 µmole of ferricyanide per minute under the conditions described above.

Inhibition assay for NCPR and NFR. The NCPR and NFR activity of the trout liver microsomes were assayed by spectrophotometer (Model UV/VIS, Unicam Ltd., Cambridge) as described previously (Masters et al., 1967). Stock aqueous solutions of DDVP were used in incubation assays and a control assay containing all the reagents except the microsomes was conducted in each experiment. For NCPR inhibition, incubation mixtures contained 0.3 M potassium phosphate buffer, pH 7.4, 1.1 mM cytochrome-c, 3.3 mM NADPH and 1.8 mg microsomal protein. The rate was measured by adding partially purified NCPR and following the formation of cytochrome-c at 550 nm. For NFR inhibition, incubation mixtures contained 0.1 M potassium phosphate buffer, pH 7.5, 5.5 mM K₃Fe(CN)₆, 3.0 mM NADH and 2.0 mg microsomal protein. The rate was measured by adding partially purified NFR and following the formation of K₃Fe(CN)₆ at 420 nm. These enzymes, in the presence or absence of DDVP, were incubated in a water bath at 25°C up to 10 min, and the reaction was started by addition of the substrate. Initial

velocity studies were conducted with cytochrome-c and $K_3Fe(CN)_6$ as the variable substrate.

Results

The amount of microsomal activity in trout liver microsomes prepared by PEG precipitation was calculated as 25.15 mg/ml. Cytochrome P450 was 0.1058 nmoles/mg protein by CO difference spectra of dithionite-reduced microsomes. NCPR activity was 0.0083 nmoles/mg protein, and the activity of NFR was 1.04 nmoles/mg protein. Partially purified NCPR and NFR was stored at $-40^{\circ}C$. The activity of enzymes did not change, even after two months. In all assays, the enzyme activities are presented as the mean of three experiments.

For NCPR, the hydroxylation rate was relatively linear up to 1.5 mg microsomal protein. Above 2.0 mg protein, the NCPR activity stabilized (Fig. 1). Therefore, microsomal incubation mixtures containing 1.5 mg/ml protein were prepared for use in all the experiments. The effect of substrate concentration on NCPR is shown in Fig. 2. The minimum amount of substrate at a stationary velocity was 0.006 mM and reduction of cytochrome-c was nearly linear up to a concentration of 0.004 mM under

the assay conditions. Kinetic analysis by Eadie-Hofstee plot showed that the enzyme had an apparent K_m of 1.04 mM (Fig. 3).

For NFR, the hydroxylation rate was approximately linear up to 2.0 mg microsomal protein. Above 2.3 mg protein, the activity for NFR was constant (Fig. 4). Therefore, microsomal incubation mixtures containing 2.0 mg/ml protein were prepared and used in all experiments. The effect of substrate concentration on NFR is displayed in Fig. 5. The minimum amount of substrate at a stationary velocity was 1.0 mM and reduction of $K_3Fe(CN)_6$ was almost linear up to a concentration of 2.0 mM under the assay conditions. Kinetic analysis by Eadie-Hofstee plot showed that the enzyme had an apparent K_m of 0.352 mM (Fig. 6).

Figs. 7 and 8 show the effect of DDVP on NCPR and NFR activity at various substrate concentrations. Maximum enzyme activity was noted at a DDVP concentration of 1.0 μM , while at higher DDVP concentrations, enzyme activity fell drastically (Table 1).

Discussion

Resistance to organophosphate pesticides has been reported for *Lepeophtheirus salmonis* (Jones et al., 1992) and insects (Bisset et

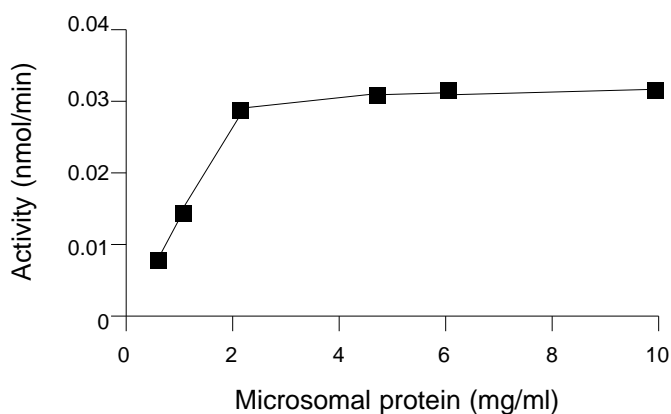


Fig. 1. Effect of microsomal protein content on microsomal NADPH-cytochrome P450 reductase (NCPR) activity in trout liver. 1 ml reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.7), 1.1 mM cytochrome-c and 3.9 mM NADPH. Data represent means \pm SD from three measurements.

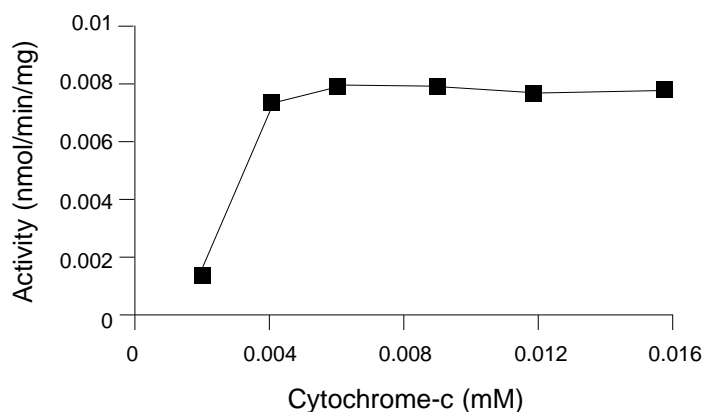


Fig. 2. Effect of substrate concentration on microsomal NADPH-cytochrome P450 reductase (NCPR) activity in trout liver. 1 ml reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.7), 3.9 mM NADPH, 1.8 mg microsomal protein and cytochrome-c. Data represent means \pm SD from three measurements.

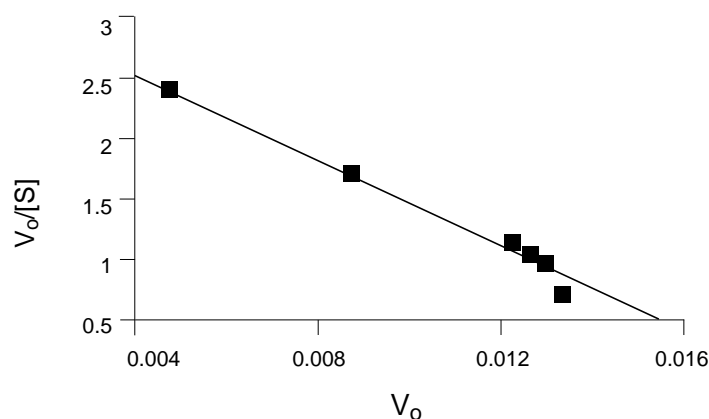


Fig. 3. Kinetics of NADPH-cytochrome P450 reductase (NCPR) activity. Each assay contained 0.1 M potassium phosphate buffer (pH 7.7), 3.9 mM NADPH, 1.8 mg microsomal protein and cytochrome-c. The Eadie-Hofstee plot of the kinetic data was used to determine the K_m and V_{max} values of the NCPR activity under optimal reaction conditions. Data represent means \pm SD from three measurements.

al., 1990). DDVP affects the nervous system by inhibiting acetylcholinesterase (WHO, 1989). It was reported that adding DDVP to a mixture of isolated hepatic microsomes stimulated lipid peroxidation in the presence of NADPH, which was inhibited by superoxide dismutase (Yamano and Morita, 1992).

Xenobiotic metabolizing NCPR and NFR were investigated in several species such as rat, sheep, camel, goat and rabbit but reports on cytochrome-c and $K_3Fe(CN)_6$ hydroxylation mechanism and the ability of aquatic animals to metabolize toxic chemicals are limited. Pollutants of major concern in the aquatic

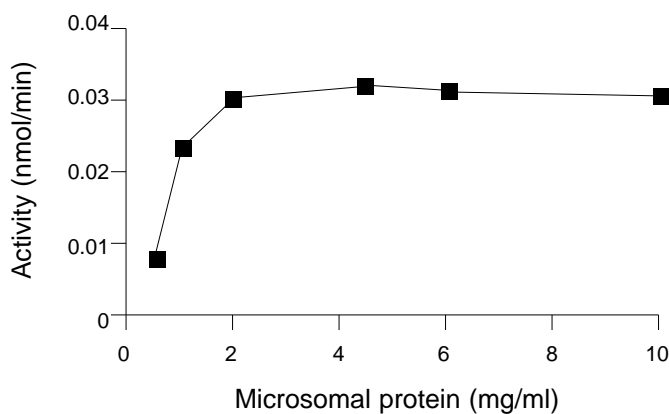


Fig. 4. Effect of microsomal protein content on microsomal NADH-ferricyanide reductase (NFR) activity in trout liver. 1 ml reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 5 mM $K_3Fe(CN)_6$ and 3mM NADH. Data represent means \pm SD from three measurements.

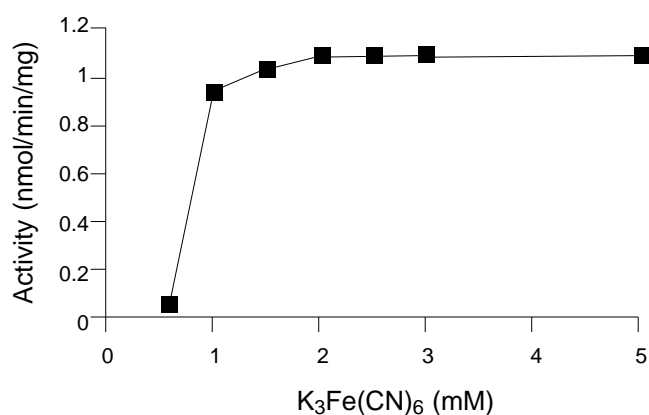


Fig. 5. Effect of substrate concentration on microsomal NADH-ferricyanide reductase (NFR) activity in trout liver. 1 ml reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 3.9 mM NADH, 2.0 mg microsomal protein and $K_3Fe(CN)_6$. Data represent means \pm SD from three measurements.

environment are aromatic hydrocarbons and their derivatives such as insecticides and herbicides. Some other chemicals may also affect biotransformation enzymes, either as inhibitors or modulators of enzyme induction or catalytic activity. DDVP has been shown to decrease enzyme activity (Yamano, 1996).

Therefore, these systems must be investigated in detail to obtain a basis for evaluating the response of biotransformation enzymes in such situations.

It was demonstrated in *Oncorhynchus mykiss* liver microsomes that DDVP inhibits NCPR and NFR. We observed that trout liver

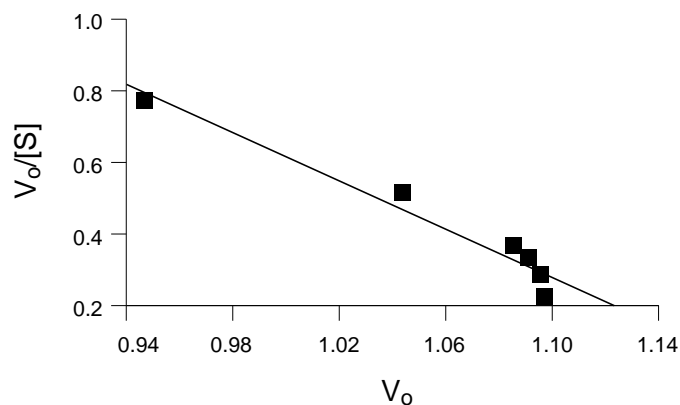


Fig. 6. Kinetics of NADH-ferricyanide reductase (NFR) activity in trout liver. Each assay contained 0.1 M potassium phosphate buffer (pH 7.5), 3 mM NADH, 2.0 mg microsomal protein and various concentrations of $K_3Fe(CN)_6$. The Eadie-Hofstee plot of the kinetic data were used to determine the K_m and V_{max} values of the NFR activity under optimal reaction conditions. Data represent means \pm SD from three measurements.

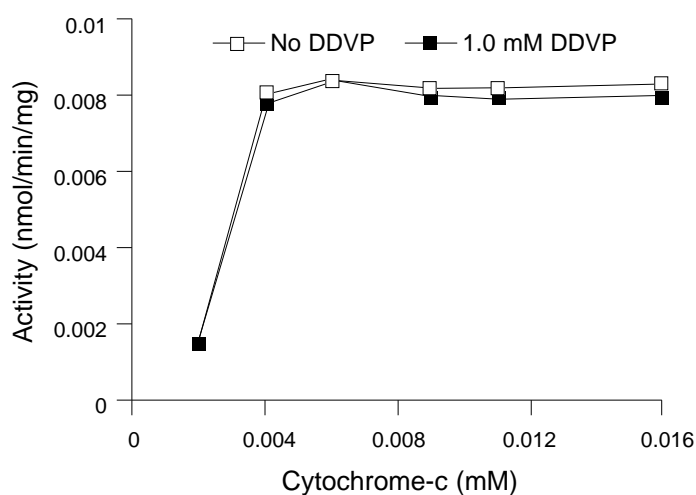


Fig. 7. 2,2-dichlorovinyl dimethyl phosphate (DDVP) inhibition of NADPH-cytochrome P450 reductase (NCPR) activity in liver microsomes. Each assay contained 0.1 M potassium phosphate buffer (pH 7.7), cytochrome-c 0.002-0.016 mM, 3.9 mM NADPH, 1.0 mM DDVP and 1.8 mg microsomal protein. Data represent means \pm SD from three measurements.

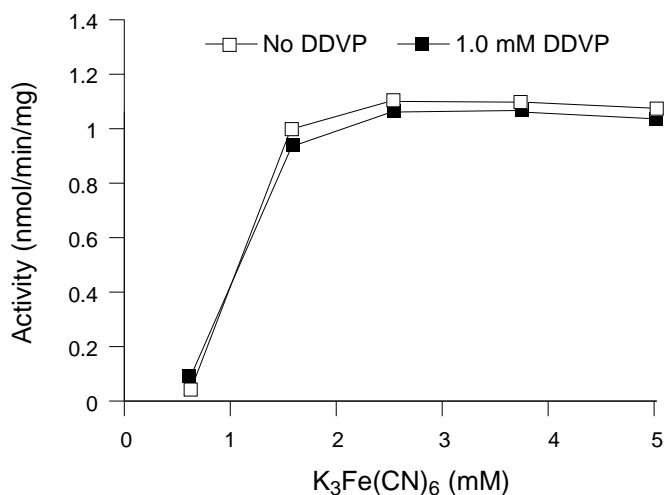


Fig. 8. 2,2-dichlorovinyl dimethyl phosphate (DDVP) inhibition of NADH-ferricyanide reductase (NFR) activity in liver microsomes. Each assay contained 0.1 M potassium phosphate buffer (pH 7.5), 0.5-5.0 mM $K_3Fe(CN)_6$, 3 mM NADH, 1.0 mM Dichlorovos and 2.0 mg microsomal protein. Data represent means \pm SD from three measurements.

Table 1. Dependence of trout liver microsomal NCPR and NFR activity on DDVP concentration.

2,2-dichlorovinyl dimethyl phosphate (DDVP; mM)	Activity (%)	
	NADPH-cytochrome P450 reductase (NCPR)	NADH-ferricyanide reductase (NFR)
1.0	100	100
5.0	99	98
10	94	83
50	89	76
100	71	52

Data represent means \pm SD from three measurements.

microsomes show activity in the presence of DDVP 1.0 mM and 2.0 mM, supporting the requirement of cytochrome-c and $K_3Fe(CN)_6$ for NCPR and NFR. NCPR hydroxylation increased up to 1.8 mg microsomal protein

content, and remained constant above 1.5 mg protein (Iyanagi et al., 1978; Arınç and Adalı 1983). It was seen that enzyme activity with maximal hydroxylation rates reached saturation at 0.005 mM cytochrome-c as the sub-

strate. NFR hydroxylation increased up to 1.5 mg microsomal protein content, and remained constant above 2.0 mg protein. The results also support that trout liver microsomes have NCPR and NFR activity (Iyanagi et al., 1978; Arinç and Adalı 1983) and that enzyme activity with maximal hydroxylation rates reached saturation at 1.0 mM $K_3Fe(CN)_6$ as the substrate. Kinetic results support that DDVP inhibition is present in the trout liver microsomes for hydroxylation of cytochrome-c and $K_3Fe(CN)_6$.

Observations in our laboratory determined inhibitory affects of DDVP on NCPR and NFR activity. There were no inhibitory effects of DDVP on NCPR and NFR activity at 1.0-5.0 mM DDVP. As the exposure of trout to such organic compound (DDVP) leads to a decrease in NCPR and NFR activity by detoxification pathways, accumulation of toxic DDVP in tissues will be affected and become increasingly hazardous to trout. On the basis of the distinct effects of organic compounds on the kinetic properties of NCPR and NFR reported above, it is speculated that a form of NCPR and NFR is inactivated by some of the DDVP species such as organophosphorus pesticides.

It has been determined that DDVP is an inactivator for the design of NADPH-cytochrome P450 reductase (NCPR) and NADH-ferricyanide reductase (NFR) inhibitors. DDVP was used for spectral interactions and inhibition by reductase. In general, this compound may influence the hem iron, however, its effectiveness as an inhibitor depends on these enzymes.

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